The homonuclear Overhauser effect in H₂O solution of low-spin hemeproteins

Assignment of protons in the heme cavity of sperm whale myoglobin

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Received September 16, 1985/Accepted in revised form December 16, 1985

Abstract. Proton-proton Overhauser effects were observed in ¹H₂O solutions of sperm whale met-cyano myoglobin. Dipolar connectivities involving hyperfine-shifted exchangeable protons such as the proximal and distal histidine ring NH's allowed us to categorize signals as arising from residues located on one side of the heme plane or on the other. With these connectivities, as well as spin-lattice relaxation times, spectral assignments were reached that were used to derive structural and dynamic information about the heme environment. Thus, it was shown that the distal histidine residue does not titrate down to pH ~ 4.1 and that the ¹CH₂ of the proximal histidine side chain tumbles with the same correlation time as the protein. Some other applications and limitations are presented.

Key words: Myoglobin, nuclear Overhauser effect, exchangeable protons, histidines

Introduction

Paramagnetic derivatives of hemoproteins such as sperm whale Mb¹ in its metcyano form present particularly interesting proton NMR spectra because the short-range electron-nucleus interactions, which cause hyperfine shifts primarily for the nuclei in the heme cavity, facilitate the resolution of active site signals from the intense diamagnetic envelope. However, the resolution improvement is accompanied by accelerated relaxation times and this second characteristic entails severe difficulties in assigning the resonances. Since peak identification is indispensable to define the heme/protein interactions from both a structural and a dynamic perspective, a number of ad hoc solutions have been applied to the spectral assignment problem.

The use of isotope labeled hemes, for example, has provided a direct way to identify the porphyrin side chain signals (Mayer et al. 1974; Krishnamoorthi 1983). While efficient and unambiguous, this method cannot yield any information on the protein matrix. The initial approach to this other aspect of the problem made use of the hyperfine shifts of paramagnetically relaxed exchangeable protons (Sheard et al. 1970). Four such signals, necessarily arising from protons located in the heme cavity, are readily resolved in mammalian metcyano myoglobins. The analysis of both their shift and relaxation behavior has resulted in the assignment of two of them to the proximal histidine (F8), and one each to the ring NH's of the distal histidine (E7) and possibly histidine FG3, stacked over pyrrole II! on the proximal side of the heme (Fig. 1). Differential paramagnetic relaxation is effective in assigning the ring NH's of the F8 and E7 histidines because the paramagnetic influence is dominant (Cutnell et al. 1981) and hence the relative spin-lattice relaxation time depends upon the relative values of R, the proton-iron distance, through

$$T_{1i}/T_{1j} = R_i^6/R_j^6.$$  

The two other hyperfine NH peaks could only be shown to be consistent with proposed assignments (Sheard et al. 1970; Cutnell et al. 1981).

The interproton Overhauser effect (NOE) has led to the identification of a few protein resonances (Johnson et al. 1983; Ramaprasad et al. 1984). As for the T₁ analysis, heme side chains provide the reference signals and the identified peaks arise from nuclei lying in the heme periphery. So far, conspicuous by their absence among assigned non-exchangeable signals are protons of the proximal

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¹ Abbreviations used: DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; Mb, myoglobin; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ppm, part per million
and distal histidines, two residues which are thought to play crucial roles in modulating the reactivity of the iron. A conceivably powerful method for assigning these non-labile proton resonances is the detection of NOE's obtained by saturation of the known labile protons from the corresponding residues or, reciprocally, the detection of NOE's to the known labile protons caused by saturation of other spins. Recent work on high-spin ferric myoglobin has shown that NOE's are observable even among strongly paramagnetically relaxed pairs of protons (Unger et al. 1985a). Although labile proton signals in myoglobin generally exhibit exchange rates with bulk water contributing to their relaxation behavior over a wide portion of the pH range, such rates may be sufficiently slow to allow for selective saturation and magnetization transfer via NOE's.

The nuclear Overhauser effect is a process occurring through space by a dipolar mechanism. In a system of two cross-relaxing spins i and j, the internuclear distance $r_{ij}$ can be calculated from the magnitude of the NOE from i to j, $\eta_{ij}$, according to the following equations (Noggle and Shirmer 1971):

$$\eta_{ij} = \sigma_{ij} T_{1j}^{-1},$$

where $T_{1j}$ is the selective $T_1$ of spin j, and $\sigma_{ij}$, the cross-relaxation rate of the pair (ij) in the slow motion limit is given by

$$\sigma_{ij} = -\left(\gamma_i^2 \hbar^2 \tau_c / (10 r_{ij}^6)\right),$$

where $\tau_c$ is the correlation time of the ij vector. The cross-relaxation rate can also be extracted from the initial slope of the NOE build-up in the truncated NOE experiment (Dobson et al. 1982). The equation used is

$$\frac{d \eta_{ij}(t)}{dt} \bigg|_{t=0} = \sigma_{ij}. $$

We present herein the results of a study based on nuclear Overhauser effects involving the four hyperfine shifted labile proton signals shown in the 24 ppm to 12 ppm window of A in Fig. 2. We provide a confirmation of earlier assignments for three signals and suggest a revision for the tentative fourth one. The identification of the two axial histidine signals permits us to assess the internal mobility of the proximal histidine and the titration behavior of the distal histidine. We also investigate the scope and limitations of the method.

Materials and methods

Myoglobin from sperm whale skeletal muscle was purchased from Sigma Chemical Co. and used without further purification. NMR experiments were performed on concentrated samples (6 mM) in 90% H$_2$O/10% $^2$H$_2$O 0.2 M in NaCl; the metcyan form was prepared by addition of excess of KCN. The pH was adjusted to the desired value by addition of 0.1 M NaOH or HCl in 90% H$_2$O/10% $^2$H$_2$O, the pH of the sample was read with a Beckman Model 3550 pH meter equipped with an Ingold microcombination electrode. The values reported for $^2$H$_2$O solutions are not corrected for isotope effect.

Proton NMR spectra were recorded in the FT quadrature mode on a Nicolet NT-500 spectrometer. The bandwidth was set to 15 kHz, 8 K or 16 K data points were collected with 16-bit digitization. The NOE experiments were performed according to

$$(A [t_1 - t_{on} - P - A c q]_n B [t_1 - t_{off} - P - A c q]_m),$$

where A and B designate two different data files, $t_1$ is a preparation time to allow the relaxation of the resonances of interest (1 to 1.5 s), $t_{on}$ is the time during which the resonance is kept saturated (typically, saturation is reached within the first 20 ms and $t_{on}$ is set to 300 ms, the power applied is about 0.2 W), $t_{off}$ is an equal time during which the decoupler is set off-resonance. P, the observe pulse was either a Redfield 2-1-4-1-2 excitation (Redfield et al. 1975) or a 1-3-3-1 pulse train (Hore 1983). In both cases some attenuation of the transmitter power was applied to obtain a $\pi/2$ pulse at the carrier (2-1-4-1-2) or to obtain a reliable 1-pulse (11°, 1-3-3-1); $n$ was set to 64 and the total number of scans in each file ($n * m$) was at least 1,024. The NOE difference spectrum was obtained by subtracting B from A. Truncated NOE experiments were performed with a $t_{on}$ (and $t_{off}$) ranging from 20 ms to 300 ms. Non-selective $T_1$ values were measured by the inversion-recovery method (Vold et al. 1968) whereas selective $T_1$ values were determined by the inversion- or saturation-recovery method. Recovery data were analyzed with a three-parameter non-linear least-squares fit. Chemical shifts are reported in parts per million with reference to DSS.

Results and discussion

Figure 1 shows a schematic representation of the histidine residues closest to the porphyrin ring (distal E7, proximal F8, and FG3). A small stretch of the F helix peptide backbone is included as well. Other pocket residues are not depicted but it should be apparent that because of steric interactions with the heme and the E7 and F8 histidines, the side chains located below the heme are several Å away from those above. Since a primary NOE is not expected between protons lying more than 6 Å apart,